

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No.

: 09/806,370

Confirmation No.: 8568

Applicant

: Holmes et al.

Filed

: October 3, 2001

TC/A.U.

: 1645

Examiner

: V. Portner

Customer No.

: 00270

Title

: MUTANT CHOLERA HOLOTOXIN AS AN ADJUVANT

Mail Stop Amendment Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450

## **DECLARATION**

Sir:

I, Mary E. Bak, residing at 1415 Comly Court, Maple Glen, PA, 19002, a citizen of the United States of America, do declare and state:

- 1. I am one of the named attorneys of record in the above-identified patent application.
- 2. This Declaration is submitted in the above-identified application in response to the Examiner's rejection under 35 USC § 112, first paragraph in the Office Action dated November 15, 2005. This Declaration is submitted in compliance with 37 C.F.R. §1.57(f) to add to the present specification the amino acid sequence of SEQ ID NO: 1, previously entered as part of the July 12, 2004 amendments by asserting that this incorporation by reference was proper and does not introduce new matter into the specification.

**EXPRESS MAIL NO: EU531733945US** 

- 3. Specifically, this Declaration is submitted to support the insertion by amendment of SEQ ID NO: 1 into the specification, which is an example of a mature wild-type cholera holotoxin subunit A sequence, as set forth in Domenighini et al... International Patent Publication No. WO 93/13202 (hereinafter Domenighini) cited in the specification at page 38, lines 10-27 and incorporated by reference. See, page 38 attached as Exhibit A herewith, where it is explicitly stated at lines 10-11: "International application WO93/13202 (36), which is incorporated by reference". Note that the Applicants referenced this publication not only to describe a series of mutations in the A subunit, but also to provide support for the nucleotide sequence encoding the A subunit of the cholera holotoxin, at page 38, lines 25-27 of the present specification, which recites "The nucleotide sequence encoding the A subunit of the cholera holotoxin is set forth in International application WO 93/13202.". This nucleotide sequence shown in Figs. 2a and 2b of Domenighini also displays the encoded mature amino acid sequence, which is illustrated in Figs. 1, 2a and 2b of <u>Domenighini</u>. Figs. 1, 2a and 2b of <u>Domenighini</u> are attached hereto as **Exhibit B**. That encoded amino acid sequence was inserted into the present specification as SEQ ID NO: 1 by way of the amendment filed on July 12, 2004. Applicants submit that the incorporation by reference of the nucleotide sequence of Figs. 2a and 2b of Domenighini is sufficient to implicitly incorporate the encoded amino acid sequence of SEQ ID NO: 1, because Figs. 2a and 2b of Domenighini also disclose the same amino acid sequence as that of Fig. 1 of Domenighini. Domenighini was cited as reference 2 in Applicants' Form PTO-1449, which was filed together with an Information Disclosure Statement on October 3, 2001, sent by Express Mail to Post Office Addressee service.
- 4. Declarant notes that a glutamic acid at amino acid position 29 of the mature A subunit of the wild-type cholera holotoxin appears in Figure 2 of Mekalanos et al., 1983, Nature, 306:551-557 (hereinafter Mekalanos), which is cited in the specification at page 2, line 4 (as Bibliography entry 1) in the context of the entire CT sequence with subunit B and 5' and 3' untranslated regions. Mekalanos was cited as reference 14 in Applicants' Form PTO-1449, which was filed together with the aforementioned Information Disclosure Statement on October 3, 2001. The mature

subunit A is indicated in Mekalanos by the number "1" appearing under the first mature amino acid "Asn" in the sequence. See, Exhibit C which is page 553 of Mekalanos, with the mature subunit A first amino acid highlighted and with the Glu at position 29 of the mature subunit A highlighted. SEQ ID NO: 1 is a mature subunit A sequence as set forth in both **Domenighini** and **Mekalanos**.

- 5. The sequence of SEQ ID NO: 1 identified in paragraph 3 above was added by way of the amendment filed on July 12, 2004. That amendment is now supported by this amended Declaration.
- 6. The sequence of SEQ ID NO: 1 identified in paragraph 3 is the previously added sequence of Domenighini. Therefore, in compliance with 37 C.F. R. §1.57(f): The material being inserted is the material previously incorporated by reference and the amendment contains no new matter.
- 7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: February 15, 2006

By: You E. Bek
Mary B. Bak

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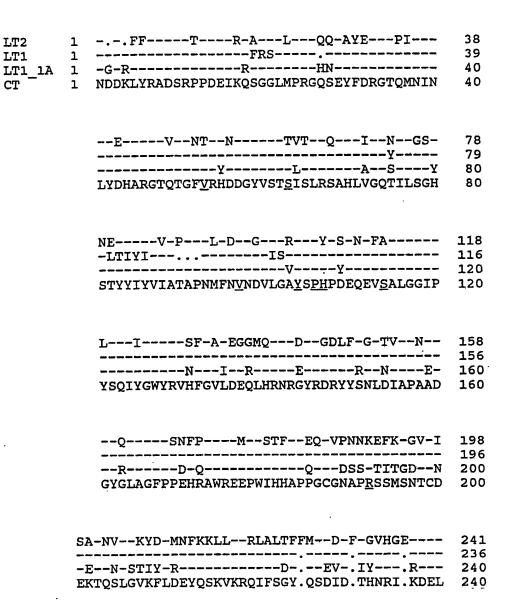
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- 38 -

acid at position 29 of the A subunit of the cholera holotoxin, in combination with a selected antigen from a pathogenic bacterium, virus, fungus or parasite, is used to prepare an antigenic composition, wherein said holotoxin enhances the immune response in a vertebrate host to said antigen.

The antigenic compositions of this invention also comprise CT-CRM containing at least one additional mutation at a position other than at amino acid residue International application WO 93/13202 (36), which is hereby incorporated by reference, describes a series of mutations in the A subunit which serve to reduce the toxicity of the cholera holotoxin. These mutations include making substitutions for the arginine at amino acid 7, the aspartic acid at position 9, the arginine at position 11, the histidine at position 44, the valine at position 53, the arginine at position 54, the serine at position 61, the serine at position 63, the histidine at position 70, the valine at position 97, the tyrosine at position 104, the proline at position 106, the histidine at position 107, the glutamic acid at position 110, the glutamic acid at position 112, the serine at position 114, the tryptophan at position 127, the arginine at position 146 and the arginine at position 192. The nucleotide sequence encoding the A subunit of the cholera holotoxin is set forth in International application WO 93/13202. International application WO 98/42375 (37) which is hereby incorporated by reference, describes making a substitution for the serine at amino acid 109 in the A subunit, which serves to reduce the toxicity of the cholera holotoxin. Therefore, using conventional techniques, mutations at one or more of these additional positions are generated.

Exhibit A to Declaration of Mary E. Bak Dated: February 15, 2006





## SUBSTITUTE SHEET

Exhibit B to Declaration of Mary E. Bak Dated: February 15, 2006

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GG	TCT	TAT	GCC	AAG	AGG	ACA	GAG	TGA	GTA	CTT	TGA	CCG	AGG	TAC	TCA	AAT	GAA	TAT	CAAC
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Figure 2a

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C G D S S R T I T G D T C N  ATCATGCACCACCAGGGTTGTGGGAATTCATCAAGAACAATCACAGGTGATACTTGTAAT H A P P G C G N A P R S S I S N T C D ATCATGCACCACGGGTTGTGGGGAATGCTCCCAAGATCATCGATCAGTAATACTTGCGAT  AGGAGACCCAGAATCTGAGGCACAATATATCTCAGGGAATATCAAAAGTTAAGAGG E T Q N L S T I Y L R E Y Q S K V K R AAAAAAACCCAAAGGTTGTGAGAGTTAAAAATTCCTTGACGAATACCCAATCTTAAAGAGA  AGATATTTCAGACTATCAGGTGTAAAAATTCCTTGACGAATACCAATCTAAAGTTAAAAGAA  AGATATTTCAGACTATCAGTCAAGAGGTTGACAATATAACAGAATTCGGGATGAATTA I F S D Y Q S E V D I Y N R I R D E L

Figure 2b

150 TOGAGICAMÁSCAAICCGAÍCCICAGICAÁAGGCTACIGÍ TGGGAAIGCÍ GICGGGACCÁAGGGGGGI TGGICGIGGGGT TI TGGIT TI TÍGITAT IGGICÁAGAIGG TÍ TI TGICACIGÍ TGGIGAIGAIGG TA TGGICA TGAIGG TA TGGICA TGAIGA TGAIGG TA TGGICA TGAIGA TGAI TOG TAGACIÁN I TEGACANTEC TATOG TOG TATOGA CONCENCAR CATE TATOGA CALCALITACIA AGGA TATOG TITA CALCAG TOTA TAGA TATOGA TOGA TATOGA TOGA TAGA CALCAG TATOGA TAGA CALCAG TAGA CAL TRECOGNITION CONTROL OF CONTROL OF CASE TO A STORY AND THE STORY CONTROL OF CASE TO CA 

Fig. 2 DNA sequence of the V. cholerae toxin operon from strain 2125. The antisense strand is shown from 5' to 3'. From nucleotide 427 to 1,663, the sequence is compared with the published sequence of LT genes<sup>20,21</sup>; elt nucleotides are shown above the sequence only where they differ from the ctx sequence except between nucleotides 810 and 830, where deletion of a T (arrowed) in the elt sequence creates a frameshift which is corrected by insertion of a C (arrowed) 16 bp downstream (see test). Analogous events have previously been seen after pseudoreversion of frameshift mutations45 but to our knowledge, this is the first naturally occurring case described. The deduced amino acid sequence of ctxA (nucleotide 516 to 1,289) and ctxB (nucleotide 1,289 to 1,660) is shown and compared with that of LT. Amino acids that differ in LT are shown below the cholera toxin amino acid sequence. In addition, for the mature B subunit sequence, differences from the published amino acid sequence of B subunit purified from strain 569B26.27 are shown in brackets. Two of these differences (amino acids 47 and 54) are also found in LT. The cleavage site between the A1 and A2 polypeptides is indicated by an arrow (amino acids 194-195). Note also the overlap of ctxA and ctxB cistrons (nucleotides 1,289-1,292). Sequence exhibiting dyad symmetry and potentially involved in transcription termination is indicated with divergent arrows. Features of the sequence immediately upstream of the ctxA gene are detailed in Fig. 3.

sequence (nucleotides 1,277-1,282, Fig. 2) of the ctxA cistron. The first two nucleotides of the ctxA translation termination signal TGA are the last two nucleotides of the ctxB translation initiation triplet ATG. This particular overlapping arrangement is also found several times in phage  $\lambda$  operons<sup>30</sup> and may be involved in translational coupling<sup>31</sup> of the ctxA and ctxB genes. However, evidence presented below suggests that this is not the case with the ctx operon. Where documented, translational coupling is observed between cistrons whose gene products interact in a one to one stoichiometry<sup>31</sup>, and in contrast, the cholera toxin molecule is composed of one A subunit and five B subunits. Moreover, E. coli produces stoichiometrically 7 times more cholera toxin B subunit than A subunit (data not shown). Fusion of the ctxB gene to various E. coli promoters allows high expression of ctxB in the absence of ctxA translational initiation signals. These data suggest that translation of ctxB relies primarily on independent initiations promoted by its own ribosome binding site.

CCCCCCACICATCACCTTCGCTGATGCGACG

Another experiment supports this conclusion. Our DNA sequencing analysis identified two NdeI sites at positions 561 and 1,337 within the ctxA and ctxB genes, respectively. The positions of these sites relative to the reading frames of ctxA and ctxB allowed us to construct a ctxA deletion which codes for an in-frame fusion of amino acid 17 of the A subunit signal sequence to amino acid 19 of the B signal and thus maintains the normal processing site of the B signal sequence (residue 21). This genetic fusion makes B subunit expression dependent on the efficiency of the A cistron translation initiation sequences, provided the hybrid signal sequence is processed at normal efficiency. NdeI digestion of plasmid pGP3 followed by ligation produced such a fusion between these two sites and gave plasmid pJM3.1. Plasmid pJM3.1 produced 0.056 µg ml<sup>-1</sup> of B subunit in E. coli MS371 while pGP3 produced 0.50 µg ml-1. These data suggest that the ctxB ribosome binding site is about ninefold more efficient than the ctxA site.

## Toxin promoter regions

1900 CE ICACTIÈRACCAGANCÉTCGGCAGCTÍGCIGNATGEÍTCIGCANGAÉTGAGCCCGTÁACA TANIGGÉGTATANTACÉCATTANGCCÉGTATGTGATÍTCGGTÁTGTÉANAATGACÁTHATTGGATÍTAITCIGATÍTCHACGG

We determined approximately 200 base pairs of sequence upstream of the XbaI sites for each of the other five additional cloned copies of the ctxA gene, cloned on plasmids pGP3, pGP4, pGP5, pGP6 and JM17. Comparison of these sequences with the corresponding region of the ctxA gene derived from strain 2,125 indicated a perfect conservation of sequence between these copies from nucleotides 413 to 590 with one notable exception. The sequence TTTTGAT comprising nucleotides 419-425, 426-432 and 433-439 of the 2,125 sequence was found tandemly repeated 3-8 times preceding different ctxA gene copies (Fig. 3). Figure 3 shows part of a sequencing gel autoradiograph that spans DNA carrying eight of these tandem repeats in the region adjacent to the cixA gene of pJM17.

To determine the position of the toxin operon promoter with respect to these repeated sequences, we used nuclease Bal31

> Exhibit C to Declaration of Mary E. Bak Dated: February 15, 2006